Thrombomodulin Regulates Keratinocyte Differentiation and Promotes Wound Healing

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The membrane glycoprotein thrombomodulin (TM) has been implicated in keratinocyte differentiation and wound healing, but its specific function remains undetermined. The epidermis-specific TM knockout mice were generated to investigate the function of TM in these biological processes. Primary cultured keratinocytes obtained from *TM*$^{lox/lox}$*; K5-Cre* mice, in which TM expression was abrogated, underwent abnormal differentiation in response to calcium induction. Poor epidermal differentiation, as evidenced by downregulation of the terminal differentiation markers loricrin and filaggrin, was observed in *TM*$^{lox/lox}$*; K5-Cre* mice. Silencing TM expression in human epithelial cells impaired calcium-induced extracellular signal–regulated kinase pathway activation and subsequent keratinocyte differentiation. Compared with wild-type mice, the cell spreading area and wound closure rate were lower in keratinocytes from *TM*$^{lox/lox}$*; K5-Cre* mice. In addition, the lower density of neovascularization and smaller area of hyperproliferative epithelium contributed to slower wound healing in *TM*$^{lox/lox}$*; K5-Cre* mice than in wild-type mice. Local administration of recombinant TM (rTM) accelerated healing rates in the TM-null skin. These data suggest that TM has a critical role in skin differentiation and wound healing. Furthermore, rTM may hold therapeutic potential for the treatment of nonhealing chronic wounds.

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INTRODUCTION

Cutaneous wound healing is a highly integrated physiological process that involves several overlapping biochemical and skin cell interactions (Werner *et al.*, 2007). This process involves the coordination of interactions among several distinct cell types, including keratinocytes, fibroblasts, endothelial cells, and inflammatory cells that emerge in wounds. Cytokines and growth factors originating from these cells, such as IL-1, transforming growth factor-β, and a variety of other growth factors (Singer and Clark, 1999) participate in the cellular interactions and have essential roles in coordinating the progress of wound healing through the regulation of cellular proliferation, migration, and differentiation. Similar to these secreted mediators, soluble thrombomodulin (sTM) may have a role in cutaneous wound healing through the regulation of cell migration and proliferation (Cheng *et al.*, 2011). However, the physiological significance of membrane-bound TM remains to be explored.

TM, a well-characterized cell surface anticoagulant glycoprotein, is expressed in endothelial cells, epidermal keratinocytes, and some other types of cells (Raife *et al.*, 1994; Esmon, 1995). TM contains five functional domains: an NH_2-terminal lectin-like region (TM domain 1 (TMD1)), an EGF-like domain (TM domain 2 (TMD2)) consisting of six EGF-like repeats, an *O*-glycosylation site–rich domain (TM domain 3 (TMD3)), a transmembrane domain (TM domain 4 (TMD4)), and a cytoplasmic tail (TM domain 5 (TMD5)), all of which exert various biological functions. In blood vessels, TM inhibits the procoagulant activity of thrombin by increasing the thrombin-dependent activation of anticoagulant protein C (Esmon and Owen, 1981). Deletion of TM lectin-like domain (TMD1) impairs the endocytic route of cells and anti-inflammatory properties in transgenic mice (Conway *et al.*, 1997, 2002). The last three EGF-like structures of TMD2 are required for protein C-activating cofactor activity and also anticoagulant activity (Zushi *et al.*, 1989). During the early phase of cutaneous wound healing, TM expression has been highly augmented in

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Abbreviations: ERK, extracellular signal–regulated kinase; HE, hyperproliferative epithelium; K5-Cre, keratin 5-Cre recombinase; MEK, MAPK/ERK kinase; RHBDL2, rhomboid-like-2; rTMD, recombinant TM domain; sTM, soluble thrombomodulin; TM, thrombomodulin; TMD, thrombomodulin domain

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the hyperproliferative epithelium (HE) in humans and mice (Peterson *et al.*, 1999), yet its function remains controversial. Keratinocyte-specific overexpression of human TM using the keratin 14 promoter did not have a significant impact on the squamous differentiation or re-epithelialization of cutaneous wounds but modulated collagen reconstitution of the wound matrix (Raife *et al.*, 1998). On the other hand, no significant differences in the rate or extent of re-epithelialization during cutaneous wound healing were observed in various TM-deficient mice such as heterozygous TM-deficient ($TM^{+/-}$), compound heterozygous ($TM^{pro/-}$), and $TM^{-/-}$ chimeric mice (Peterson *et al.*, 1999). The ectodomain of TM protein, which includes the EGF-like domain (TMD2), has mitogenic activity toward fibroblasts and also promotes angiogenesis (Hamada *et al.*, 1995; Shi *et al.*, 2005). Our recent study suggested that TM ectodomains are shed by a rhomboid intramembrane serine protease, rhomboid-like-2 (RHBDL2), during the early phase of wound healing, and the TM ectodomain appears to be an autocrine/paracrine factor in wound healing (Cheng *et al.*, 2011). Because 3,4-dichloroisocoumarin, a rhomboid intramembrane serine protease inhibitor, suppresses TM ectodomain shedding and wound healing, sTM released by RHBDL2 is considered essential for proper wound healing. Nevertheless, a definite role for sTM during the wound healing process is difficult to establish because of the possible nonspecific inhibitory effects of 3,4-dichloroisocoumarin in this process. In addition, the function of the membrane-bound TM in the neoepidermis has rarely been investigated in previous studies.

To avoid embryonic lethality that occurs following TM knockout, and to specifically explore the functions of epithelial TM in cell differentiation and the wound healing process, skin-specific TM-null mice with conditional disruption of TM were generated using the *Cre-loxP* recombination technology. These tissue-specific TM-deleted mice were used to reappraise the role of TM and its contribution to cutaneous wound healing. We bred these $TM^{lox/lox}$ mice with *keratin 5-Cre recombinase* (*K5-Cre*) transgenic mice (Isermann *et al.*, 2001; Liang *et al.*, 2009), instead of using a cell model, to produce $TM^{lox/lox}$*; K5-Cre* mice. This study may help clarify the role of epithelial TM, especially the membrane-bound form, on differentiation and wound healing.

RESULTS

Abrogation of TM expression causes abnormal differentiation of mouse keratinocytes

To verify the successful generation of skin-specific TM knockout mice, TM expression was analyzed in the epidermis of $TM^{lox/lox}$*; K5-Cre* mice. The results of reverse transcriptase–PCR and western blotting confirmed that *Cre*-mediated recombination abolishes TM expression at the mRNA and protein levels in the epidermis (Figure 1a and b). It has been shown that calcium induces TM expression in the primary cultured keratinocytes harvested from the mouse tail skin (Caldelari *et al.*, 2000). In our study, incubation of keratinocytes from $TM^{lox/lox}$ mice with 1.2 mM calcium induced strong TM expression, whereas those from $TM^{lox/lox}$*; K5-Cre* mice did not express TM under similar conditions. In addition, the adhesion molecule E-cadherin localized to the cell junctions of keratinocytes from $TM^{lox/lox}$ mice, but not to those from $TM^{lox/lox}$*; K5-Cre* mice (Figure 1c). Moreover, calcium could not trigger the upregulation of epidermal differentiation markers on primary cultured keratinocytes from $TM^{lox/lox}$*; K5-Cre* mice (Figure 1d).

Hematoxylin and eosin staining of tissue sections obtained from mouse tails of $TM^{lox/lox}$ and $TM^{lox/lox}$*; K5-Cre* mice skin showed significant differences (Figure 2a and b). The epidermal keratinocytes of $TM^{lox/lox}$*; K5-Cre* mice showed few flat nuclei, which are characteristic of terminal differentiation (Figure 2c and d). TM expression was undetectable in the $TM^{lox/lox}$*; K5-Cre* mice compared with $TM^{lox/lox}$ mice (Figure 2e and f). The expression of markers of terminal differentiation in keratinocytes, namely, loricrin and filaggrin, was downregulated in TM-null skin (Figure 2g–j). Taken together, these results suggested that TM has a role in keratinocyte differentiation and cell–cell adhesion.

TM silencing impairs calcium-induced extracellular signal–regulated kinase (ERK) phosphorylation and keratinocyte differentiation

In this study, stable TM knockdown HaCaT cells (Figure 3a) were used to investigate the role of TM in calcium-triggered differentiation and the underlying molecular mechanism. Consistent with previous studies showing that calcium induces keratinocyte differentiation through the activation of Ras-independent Raf/MEK/ERK signaling (Schmidt *et al.*, 2000), calcium treatment caused ERK phosphorylation in parental HaCaT and vector-transfected cells. In TM-silenced TM short hairpin RNA cells, however, calcium treatment failed to induce ERK phosphorylation at 30 minutes or upregulation of loricrin at 24 hours (Figure 3b and c). These results suggested that TM expression in keratinocytes has a critical role in calcium-induced keratinocyte differentiation and which may through the ERK pathway.

TM knockout keratinocytes exhibit decreased spreading and slow migration ability

Spreading on a flat surface is one of the important characteristics of epithelial cell differentiation (Micallef *et al.*, 2009). To further investigate the role of TM in cell differentiation, primary keratinocytes from transgenic mice were cultured in high calcium medium (1.2 mM) for 24 hours under a low cell density condition. The spreading area of each cell was ~75% smaller in primary keratinocytes derived from $TM^{lox/lox}$*; K5-Cre* mice than in those from their $TM^{lox/lox}$ littermates (Figure 4a and b). Our previous studies demonstrated that TM functions as a cell adhesion molecule and is an ezrin-interacting protein that promotes collective cell migration. Here, a wound healing assay with primary cultured keratinocytes was performed to assess cell migration ability. The results showed a slower healing response in cells from $TM^{lox/lox}$*; K5-Cre* mice than in those from $TM^{lox/lox}$ (Figure 4c), with a recovery rate of approximately one-sixth of that observed in the $TM^{lox/lox}$ mice (Figure 4d). Taken together, these data suggested that membrane-bound TM in keratinocytes contributes to cell differentiation and migration.

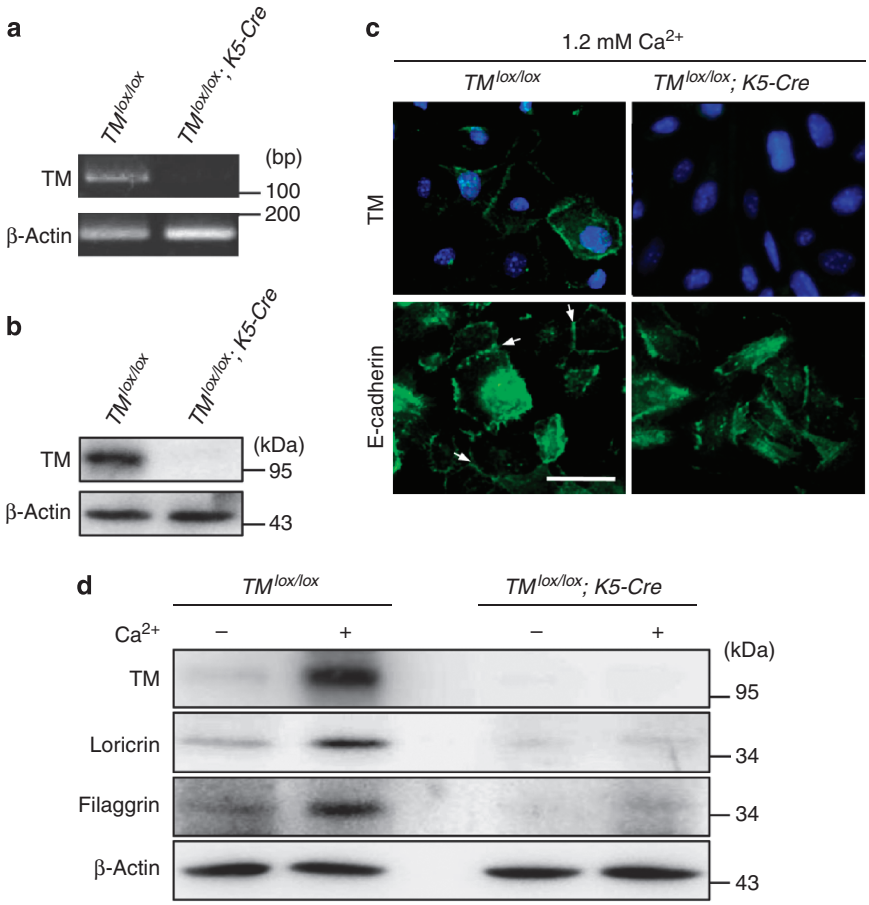

Figure 1. Tissue-specific recombination in *TM$^{lox/lox}$; K5-Cre* mice. Tissue-specific deletion of thrombomodulin (TM) was detected at skin epidermis from adult mouse by (**a**) reverse transcriptase–PCR (RT–PCR) and (**b**) western blot analysis. (**c**) Immunofluorescence staining of TM and E-cadherin in primary cultured keratinocytes (passages 3–5) from adult *TM$^{lox/lox}$; K5-Cre* mice and *TM$^{lox/lox}$* mice after culture in 1.2 mM calcium medium for 96 hours. Arrows indicate the local expression of E-cadherin at cell–cell contacts. Bar = 100 μm. (**d**) Detection of calcium-induced differentiation markers in cultured keratinocytes by western blotting. K5-Cre, keratin 5-Cre recombinase.

Knockout of TM in the epidermis delays wound recovery *in vivo*

The expression of TM is highly enhanced in the HE during the early phases of cutaneous wound healing in humans and mice (Peterson *et al.*, 1999). The *in vivo* effects of TM expression in keratinocytes were examined using a wound healing model in mice, in which a full-thickness transdermal incision was made along the dorsal spine and the rate of wound closure was measured. Wound closure was significantly delayed in TM knockout mice compared with their *TM$^{lox/lox}$* littermates (Figure 5a and b). In addition, intense neovascularization was observed near the incision site in *TM$^{lox/lox}$* mice 6 days after injury, whereas the wound region of *TM$^{lox/lox}$; K5-Cre* mice showed significantly less neovascularization (Figure 5c). The number of branching points in dermal blood vessels at the incision was analyzed using a dissecting microscope, which showed a 50% reduction of branching points in *TM$^{lox/lox}$; K5-Cre* mice compared with that in *TM$^{lox/lox}$* mice (Figure 5d). Histological sections of the wound area obtained 3 and 5 days after injury were examined. On day 5, the area of HE was ~50% smaller in *TM$^{lox/lox}$; K5-Cre* mice than in *TM$^{lox/lox}$* mice (Figure 5e and f). These results suggested that TM may be involved in several processes associated with the formation of HE and cutaneous wound healing, such as neovascularization and epithelial proliferation.

Recombinant TM accelerates cutaneous wound healing in *TM$^{lox/lox}$; K5-Cre* mice

Our previous study indicated that sTM released by serine protease RHBDL2 is one of the key molecules involved in cutaneous wound healing. An analysis of tissue extracts and the skin culture medium 5 days after the incision showed lower levels of TM in the tissue extract and less sTM released from the skin samples of *TM$^{lox/lox}$; K5-Cre* mice than from those of *TM$^{lox/lox}$* mice (Figure 6a), suggesting that reduced sTM might contribute to delayed wound healing in the TM-null epidermis. Therefore, we tested whether TM supplementation might accelerate wound healing in *TM$^{lox/lox}$; K5-Cre* mice. Local intradermal injection of human recombinant TM containing all the ectodomains of TM (rTMD123) significantly improved the rate of wound healing (Figure 6b). The western blot analysis showed the existence of injected human rTMD123 for at least 48 hours after the last injection

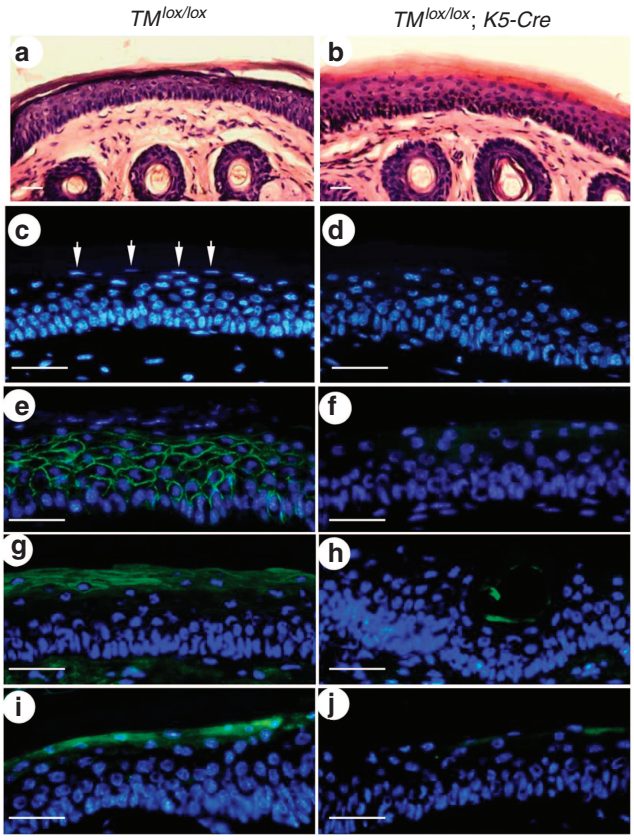

Figure 2. Atypical epidermal differentiation is shown in *TM$^{lox/lox}$; K5-Cre* mice. The cross-sections from adult mice tail were stained with (**a**, **b**) hematoxylin and eosin (H&E), (**c**, **d**) 4′,6-diamidino-2-phenylindole (DAPI), (**e**, **f**) thrombomodulin (TM), and differentiation markers such as (**g**, **h**) filaggrin and (**i**, **j**) loricrin. Arrows indicate flattened cell nucleus. All bars = 100 μm. K5-Cre, keratin 5-Cre recombinase.

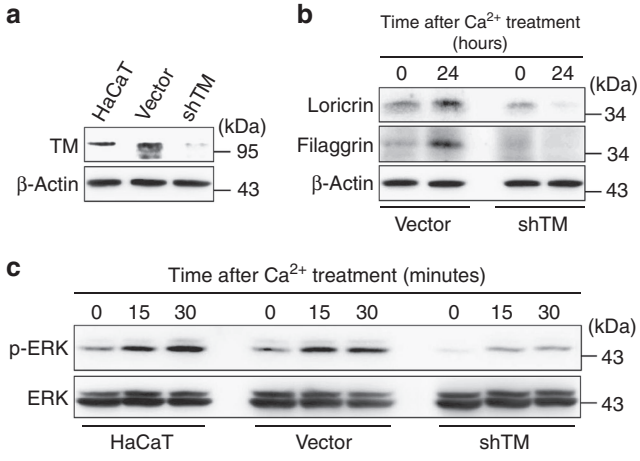

Figure 3. Silencing of thrombomodulin (TM) expression inhibits the calcium-induced extracellular signal–regulated kinase (ERK) phosphorylation and terminally differentiated marker expression in human keratinocytes. (**a**) Immunoblotting of TM expression in stable transfected HaCaT cells (passages 20–30). (**b**, **c**) Western blot analysis of calcium-triggered phosphorylation of ERK (p-ERK) signaling (**b**) and expression of keratinocyte differentiation markers (**c**) in stable transfected HaCaT cells at indicated time points. Representative results from three independent experiments are shown. shTM, TM short hairpin RNA.

(Figure 6c). Furthermore, administration of mouse rTMD23, an angiogenic factor (Shi *et al.*, 2005), caused a similar enhancement of cutaneous wound healing as rTMD123 in *TM$^{lox/lox}$; K5-Cre* mice (Figure 6d and e).

DISCUSSION

Skin injury promotes TM expression in the neoepidermis (Peterson *et al.*, 1999), suggesting that TM has a role in wound healing and epidermis development. To unveil the functions of epithelial TM, several heterozygous transgenic mice and keratinocyte-specific TM overexpression mice have been generated because TM knockout in mice results in lethality (Healy *et al.*, 1995; Raife *et al.*, 1998; Peterson *et al.*, 1999). In our previous work, skin injury induced the expression of rhomboid serine protease RHBDL2, which caused the release of sTM, a possible intrinsic factor promoting wound healing (Cheng *et al.*, 2011). In this study, keratinocyte-specific TM knockout mice that did not show physiological differences from control mice were generated to further examine the role of TM in the wound healing process. The *Cre-loxP* strategy resulted in a significant abolishment of TM expression detected by reverse transcriptase–PCR and western blotting in transgenic keratinocytes, and TM expression in these keratinocytes was not enhanced following incubation with calcium (1.2 mM). TM expression appears to be a consistent feature of squamous differentiation, as TM staining is positively observed in stratified squamous epithelia of various tissues, including skin, and oral mucosa, and in squamous metaplasia of nonstratified epithelia (Raife *et al.*, 1994). It was demonstrated that E-cadherin is required for calcium-induced keratinocyte differentiation (Tu *et al.*, 2008). In this study, we demonstrated that TM-null skin exhibited striking downregulation of loricrin and filaggrin in stratified keratinizing epithelia. Downregulation of these two major protein markers in differentiating keratinocytes in the granular cells and stratum corneum of stratified keratinizing epithelia indicates that the differentiation process is compromised in the TM-null keratinocytes. Similarly, our data showed that calcium (1.2 mM) failed to promote the expression of epidermal differentiation markers in TM-null mouse keratinocytes and TM-silenced human HaCaT cells, suggesting that membrane-bound TM may participate in keratinocyte differentiation process directly. In addition, loss of TM expression in primary keratinocytes altered the location of E-cadherin at cell–cell junction, suggesting that both TM and E-cadherin may function synergistically in the process of keratinocyte differentiation.

Consistent with previous studies showing that activation of the Raf/MEK/ERK pathway has a crucial role in extracellular calcium-induced keratinocyte differentiation (Schmidt *et al.*, 2000), we found that stable silencing of TM expression inhibits ERK phosphorylation and cell differentiation. Intracellular molecules, such as Rho or E-cadherin, are also required for calcium-induced keratinocyte differentiation (Tu *et al.*, 2008, 2011). These molecular pathways may probably be involved in TM-regulated epithelial differentiation.

Our previous studies suggested that TM may function as a calcium-dependent cell adhesion molecule (Huang *et al.*,

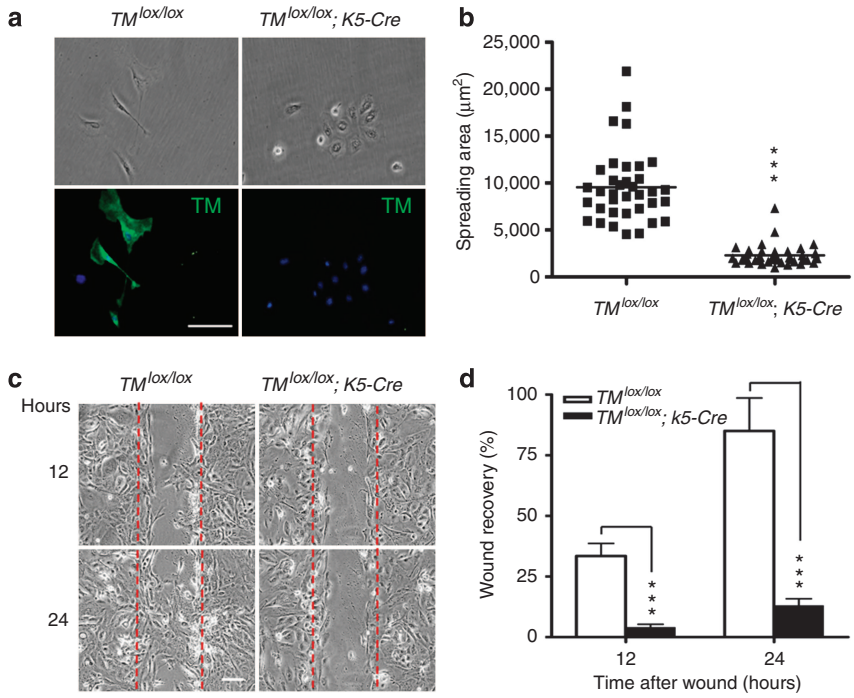

Figure 4. Reduction of spreading area and scratch-wound migration in primary cultured keratinocytes derived from *TM*$^{lox/lox}$; *K5-Cre* mice. (**a**) Immunostaining of thrombomodulin (TM) and (**b**) quantitative analysis of spreading area in primary keratinocytes ($n=40$) from *TM*$^{lox/lox}$and *TM*$^{lox/lox}$; *K5-Cre* mice. (**c**) Scratch-wound migration assay on confluent primary keratinocytes (passages 3–5). Red dotted lines indicate the wound borders at beginning. (**d**) Quantitative representation of the results of **c**. Data are from three independent experiments. ***$P<0.001$. All bars = 100 μm. K5-Cre, keratin 5-Cre recombinase.

2003). In this study, the spreading areas of TM knockout keratinocytes were significantly smaller than those of the controls. Although keratinocyte-specific overexpression of human TM did not affect normal squamous differentiation or re-epithelialization of cutaneous wounds in previous studies (Raife *et al.*, 1998), our results showed that TM-null keratinocytes had reduced cell migration ability in cell culture within 24 hours, suggesting that membrane-bound TM may have a role in cell migration. In addition, reduction of neovascularization and epithelial proliferation near the wound area was also observed in TM knockout skin. These findings confirm our previous results showing that TM domains or sTM may serve as an angiogenic and mitogenic factor for endothelial and epithelial cells (Shi *et al.*, 2005; Cheng *et al.*, 2011).

The sTM fragments in plasma and urine are proposed to be released from the cell surface by proteases and may serve as diagnostic biomarkers of blood circulation disorders such as coronary heart disease, or diabetes (Ishii and Majerus, 1985; Gabat *et al.*, 1996; Blann and Yip, 1998; Nakano *et al.*, 1998; Salomaa *et al.*, 1998, 1999; Thorand *et al.*, 2007). In this study, reduction of sTM and decreased TM levels were observed in primary cultured skin from *TM*$^{lox/lox}$; *K5-Cre* mice, and these mice exhibited delayed cutaneous wound healing. Furthermore, treatment with recombinant TM fragments (either mice rTMD23 or human rTMD123) enhanced skin wound healing in *TM*$^{lox/lox}$; *K5-Cre* mice. A high degree of homology between human and mouse TM was detected by protein alignment. Therefore, either human or mouse TM fragments should be effective in promoting wound healing in a mouse model. Our results suggest that TM in the neoepidermis may have at least dual roles in wound healing. The membrane-bound TM of keratinocytes is proposed to be involved in the regulation of cell differentiation and migration. On the other hand, the keratinocytes of the HE may serve as the major source of sTM, which act as a paracrine factor to modulate angiogenesis at the wound site and promote wound healing.

Angiogenesis is an essential step of the wound healing process, as new blood vessels are required for the supply of oxygen and nutrients, debris removal, and granulation tissue formation (Singer and Clark, 1999). Impaired skin blood flow is a major pathologic event in diabetic wounds (Singer and Clark, 1999; Khan *et al.*, 2000). Our study, using tissue-specific knockout mice, suggests that keratinocyte-specific TM may be a critical regulator of keratinocyte differentiation and cutaneous wound healing. Moreover, local treatment with rTM fragments improved skin wound healing probably by promoting neovascularization and epithelial proliferation. Therefore, rTM might hold therapeutic potential in the treatment of chronic wounds such as diabetic ulcers. In conclusion, our study indicated that membrane-bound TM has a critical role in epithelial differentiation and sTM holds promise in promoting wound healing.

MATERIALS AND METHODS

Reagents

Anti-E-cadherin, antifilaggrin, and antiloricrin antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-TM, p-ERK, ERK,

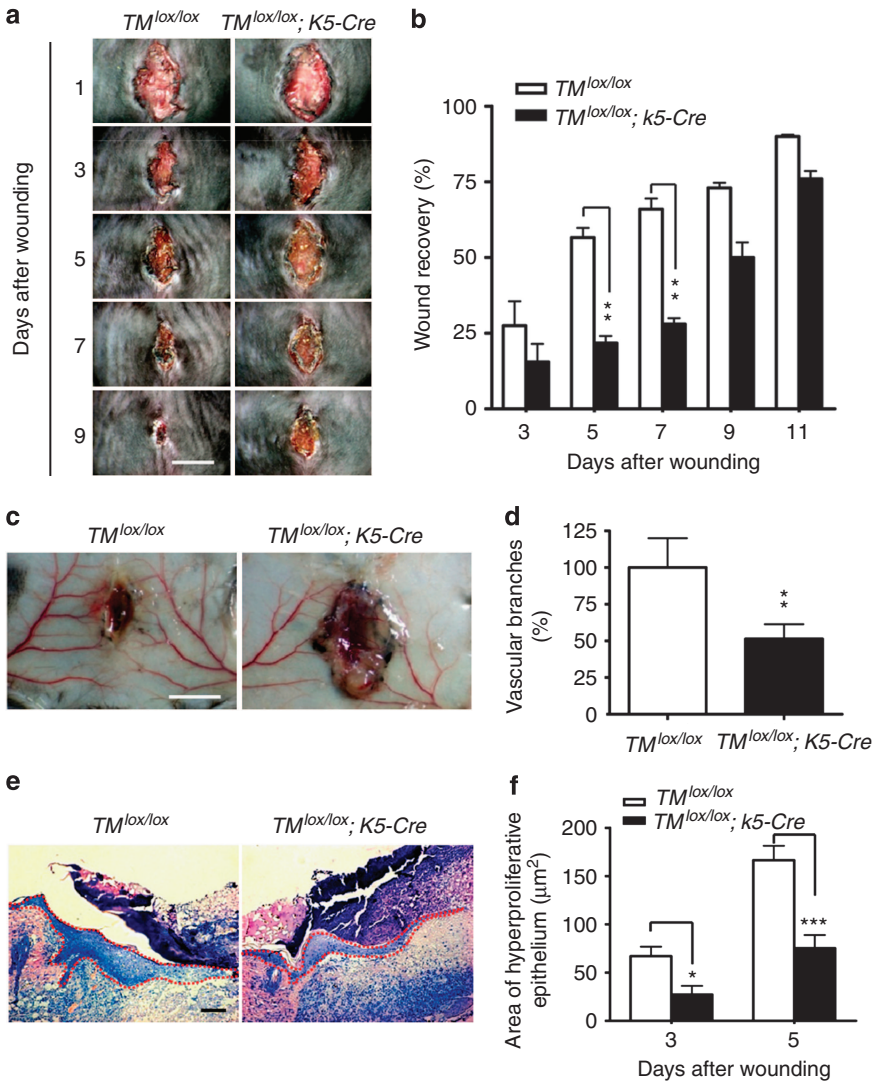

Figure 5. Reduction of cutaneous wound healing, neovascularization, and epithelial proliferation in *TM*$^{lox/lox}$; *K5-Cre* mice. (**a**) Macroscopic observation of wound healing in transgenic mice that were photographed at indicated time points after injury. (**b**) Quantitative representation of **a**. (**c**) Vascularization around the wound of the skin under site was observed at day 6 after injury. (**d**) Quantitative representation of the blood vessel branches from **c**. (**e**) Sections from the middle of the wound were stained with hematoxylin and eosin (H&E) at day 6 after injury. (**f**) Quantitative representation of the results of **e** at indicated time points. The red dotted lines mark the hyperproliferative epithelium (HE). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ ($n=5$). Bar = 1 cm (**a**, **c**). Bar = 100 μm (**e**). K5-Cre, keratin 5-Cre recombinase.

glyceraldehyde-3-phosphate dehydrogenase, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody against mouse rTMD23 was generated by a local biotech company and purified with an affinity column of rTMD23-conjugated Sepharose gel (Sigma Chemical, St Louis, MO). The keratinocyte serum-free medium was purchased from Invitrogen Life Technologies (Invitrogen, Carlsbad, CA).

Mouse strains

The conditional *TM* knockout mouse model (*TM*$^{lox/lox}$) was generated with Cre-recombinase systems described previously (Isermann *et al.*, 2001) and kindly provided by Dr Hartmut Weiler (Blood Research Institute, The Blood Center of Wisconsin and the Medical College of Wisconsin, Milwaukee, WI). The *K5-Cre* transgenic mice were kindly provided by Dr Chun-Ming Chen at the National Yang-Ming University (Taipei, Taiwan, Republic of China) (Liang *et al.*, 2009). The keratinocyte-specific TM deletion mice were generated by crossing *TM*$^{lox/lox}$ mice with the *K5-Cre* transgenic mice. The TM expression levels of offspring were analyzed by reverse transcriptase–PCR and western blot analysis. All mouse lines were maintained by backcrossing to C57BL/6J. All animal experiments were approved by the Institutional Animal Care and Use Committee of National Cheng Kung University, Tainan, Taiwan.

Murine keratinocyte primary culture

Primary keratinocytes were isolated from the mice tail skin and cultured by using a modified protocol (Caldelari *et al.*, 2000; Lichti *et al.*, 2008). In brief, the sterilized tail skins from mice were incubated with dispase overnight at 4 °C, and the epidermis was peeled carefully. After trypsinization of the epidermis, primary

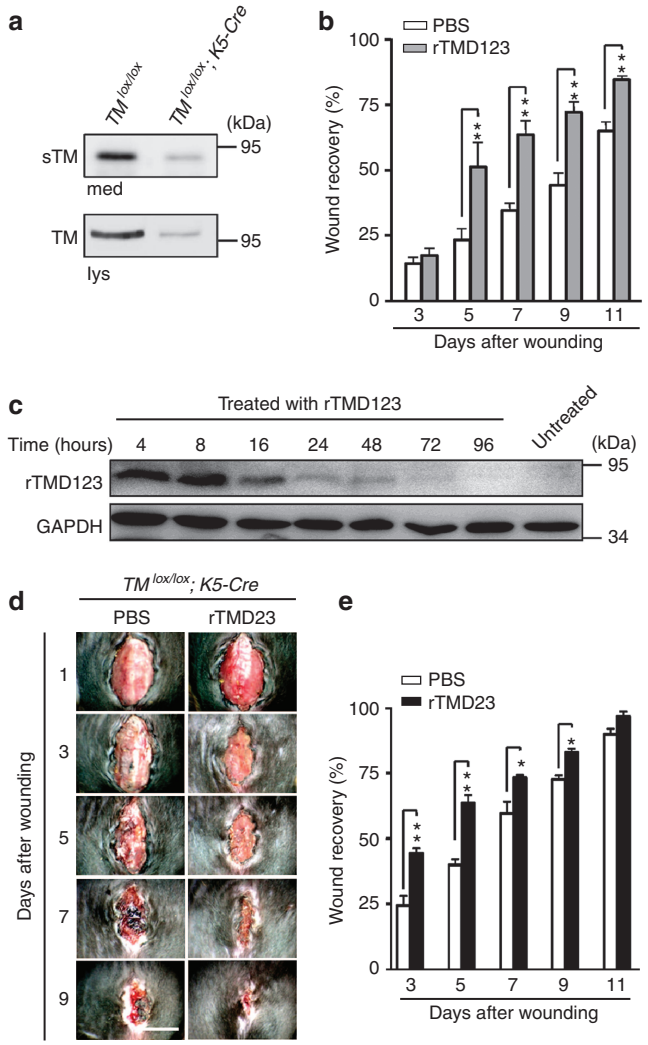

Figure 6. Recombinant thrombomodulin (TM) fragments recover the delayed skin wound healing in $TM^{lox/lox}$; *K5-Cre* mice. (**a**) Western blot analysis of soluble thrombomodulin (sTM) in concentrated conditional medium (med) and TM protein in primary cultured skin lysate (lys) from $TM^{lox/lox}$ and $TM^{lox/lox}$; *K5-Cre* mice, separately. (**b**) Quantitative results of mice skin wound recovery when treated with human rTMD123 (recombinant TM containing all the ectodomains of TM) by subcutaneous injection. (**c**) Existence of rTMD123 at indicated time points after the last injection. (**d**) Macroscopic observation of wound recovery in the treatment of mice rTMD23 on TM knockout skin. (**e**) Quantitative representation of the results of **c**. $*P<0.05$, $**P<0.01$ ($n=5$). Bar = 1 cm. K5-Cre, keratin 5-Cre recombinase.

keratinocytes were collected and cultured with keratinocyte serum-free medium in type IV collagen–coated dishes.

Cell spreading assay

Primary keratinocytes were seeded in type IV collagen (20 μg ml^{-1}; BD Biosciences, Sparks, MD)–coated six-well plates. After 1-hour incubation at 37 °C, nonattached cells were washed out by phosphate-buffered saline and attached cells were fixed with 4% formaldehyde. After staining with TM, multiple fields from each treatment condition were photographed, and cell spreading areas were measured by Metamorph software (Universal Imaging, Downingtown, PA).

In vitro wound healing assay

As described previously, the cells were photographed using a time-lapse video microscopy system (Olympus, Tokyo, Japan) immediately after wounds were made (Cheng *et al.*, 2011). The rate of wound recovery was quantitatively determined with ImageJ software (National Institutes of Health, Bethesda, MD). The percentage of wound recovery at the indicated time points was calculated by comparing it with the original wound area.

Stable transfected HaCaT cells

Silencing *TM* gene expression in HaCaT cells was accomplished using short hairpin RNA technology with the pSM2c vector system (GenDiscovery Biotechnology, New Taipei, Taiwan) (Kao *et al.*, 2010). HaCaT cells were transfected with the short hairpin RNA expression construct or with empty vector and selected with 1 μg ml^{-1} puromycin.

Reverse transcriptase–PCR

Total RNA was isolated from cultured keratinocytes using the TRIzol reagent (Invitrogen). Total RNA (1 μg) and oligo(dT) primer (Promega, Madison, WI) were used for the complementary DNA synthesis. PCR primers for TM and actin were: TM forward, 5′-CTTGTGCAATAGG AGCACGA-3′ and TM reverse, 5′-GACACAAAAATGCTCGCAGA-3′; and actin forward, 5′-TGTTACCAACTGGGACGACA-3′ and actin reverse, 5′-GGGGTGTTGAAGGTCTCAAA-3′.

Western blot analysis

Approximately 50 μg total protein was separated in a 10% SDS–polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. After probing with a primary and a secondary antibody, the signal was detected using an enhanced chemiluminescence reagent (Amersham Phamacia Biotech, Piscataway, NJ).

Histology

Tissue samples were fixed in 4% formaldehyde, embedded in paraffin, and cut into 5-μm sections. For histological analysis, sections were stained with hematoxylin and eosin or the indicated antibodies. Secondary antibodies conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) were used for immunofluorescence staining.

Expression and purification of rTMD proteins

The pPICZαA and pCR3 vectors (Invitrogen) were used for the expression of mouse rTMD23 and human rTMD123 in the *Pichia pastoris* and HEK293, separately (Shi *et al.*, 2005). DNA fragments encoding rTMD23 and rTMD123 were obtained as described previously (Han *et al.*, 2000). Conditioned medium containing expressed mouse or human rTMD proteins were applied to a nickel-chelating Sepharose column (Amersham Pharmacia Biotech AB), and eluted under sterile conditions.

In vivo cutaneous wound healing assay

This assay was performed as described previously with modifications (Tsuboi and Rifkin, 1990). The skin surrounding the wound was treated with injections of the rTMD protein (125 μg kg^{-1}). This treatment was applied to the wounds once per day at days 2 and 3 after injury. The cut edge of each wound was photographed at the indicated time points after surgery for analysis of wound closure using ImageJ software. The percentage of daily wound closure was

compared with the original wound area on day 1 after the wound generation (Greenhalgh *et al.*, 1990). The wound margins were harvested to detect the retention of injected rTMD protein by western blotting with the antibody to recognize human TM.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was analyzed using one-way analysis of variance and Student's *t*-test. The *P*-values of <0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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